

AD-A054 653 ARMY MEDICAL RESEARCH INST OF INFECTIOUS DISEASES FR--ETC F/G 6/1
BIOLOGICAL ACTIVITIES OF THE PEPTIDES OF STAPHYLOCCOCAL ENTEROT--ETC(U)
MAY 78 L SPERO, B A MORLOCK

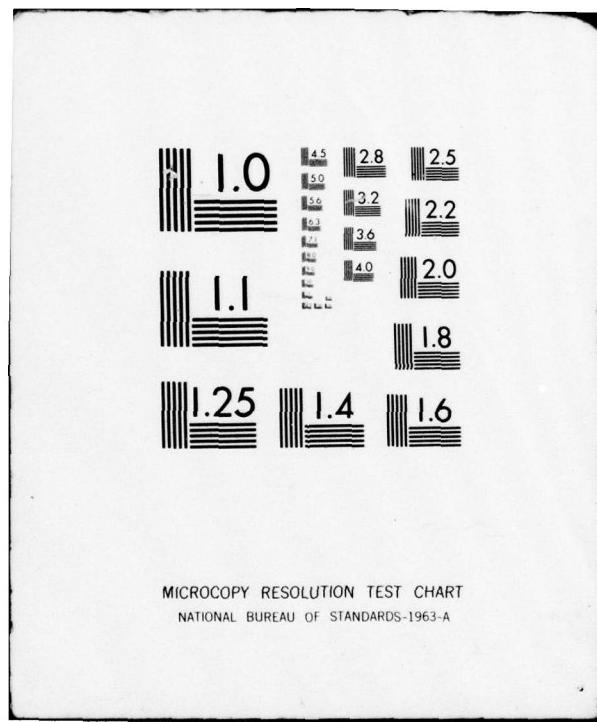
UNCLASSIFIED

NL

| OF |
AD
A054653
FILE NUMBER



END
DATE
FILED
6 -78
DDC



AD A054653

UNCLASSIFIED

SECURITY CLASSIFICATION OF THIS PAGE (When Data Entered)

REPORT DOCUMENTATION PAGE		READ INSTRUCTIONS BEFORE COMPLETING FORM
1. REPORT NUMBER 6	2. GOVT ACCESSION NO.	3. RECIPIENT'S CATALOG NUMBER H-11
4. TITLE (and Subtitle) Biological Activities of the Peptides of Staphylococcal Enterotoxin C Formed by Limited Tryptic Hydrolysis .		5. TYPE OF REPORT & PERIOD COVERED Interim rept., Celt
6. AUTHOR(s) Leonard/Spero Beverly A./Morlock		7. CONTRACT OR GRANT NUMBER(s) 12APP
9. PERFORMING ORGANIZATION NAME AND ADDRESS U.S. Army Medical Research Institute of Infectious Diseases, Fort Detrick, Frederick MD 21701		10. PROGRAM ELEMENT, PROJECT, TASK AREA & WORK UNIT NUMBERS 61102 3M161102BS03-00-006
11. CONTROLLING OFFICE NAME AND ADDRESS U.S. Army Medical Research and Development Command, Office of the Surgeon General Department of the Army, Fort Detrick, MD 21701		12. REPORT DATE 11/8 May 78
14. MONITORING AGENCY NAME & ADDRESS (if different from Controlling Office) 1233P		13. NUMBER OF PAGES 26 pages, 4 figures
		15. SECURITY CLASS. (of this report) UNCLASSIFIED
		15a. DECLASSIFICATION/DOWNGRADING SCHEDULE
16. DISTRIBUTION STATEMENT (of this Report) Approved for public release; distribution unlimited.		
17. DISTRIBUTION STATEMENT (of the abstract entered in Block 20, if different from Report) DDC DISTRIBUTION E		
18. SUPPLEMENTARY NOTES Reprints bearing assigned AD number will be forwarded upon receipt. To be submitted for publication in The Journal of Biological Chemistry.		
19. KEY WORDS (Continue on reverse side if necessary and identify by block number)		
20. ABSTRACT (Continue on reverse side if necessary and identify by block number) Staphylococcal enterotoxin C is converted to a doubly cleaved molecule by trypsin digestion with one of the scissions internal to the disulfide loop and one external to it [J. Biol. Chem. 251, 5580-5588 (1976)]. The larger, disulfide-containing polypeptide (M_r = 22,000) exhibited excellent binding to antiserum to the intact enterotoxin. The residual amino terminal fragment (M_r = 6,500) also bound to this antibody but only weakly. Only the carboxyl terminal carboxamidomethylated moiety of the 22,000 M_r polypeptide (M_r = 19,000)		

AB

UNCLASSIFIED

SECURITY CLASSIFICATION OF THIS PAGE(When Data Entered)

combined with anti-enterotoxin C₁. Both the 22,000 M_r and 6,500 M_r polypeptides could partially inhibit the binding of enterotoxin C₁ to its antibody in a competitive system. It is suggested that enterotoxin C₁ possesses three major antigenic determinants, two on Cam 19,000 and one on the 6,500 M_r fragment. A significant degree of refolding to a native-like conformation is indicated for the 22,000 M_r and the Cam 19,000 materials by (a) the strong binding of these polypeptides to anti-enterotoxin C₁; (b) the strong binding of enterotoxin C₁ to antibody to the 22,000 M_r polypeptide; and (c) their circular dichroic spectra in the far-ultraviolet.

The 6,500 M_r polypeptide exhibited mitogenic activity but not emetic activity. Conversely the 22,000 M_r polypeptide was able to induce diarrhea in rhesus monkeys, but was not mitogenic suggesting that the active sites for these activities are widely separated on the enterotoxin molecule.

ACCESSION for		
NTIS	White Section	<input checked="" type="checkbox"/>
DDC	Buff Section	<input type="checkbox"/>
UNANNOUNCED		<input type="checkbox"/>
JUSTIFICATION.....		
BY.....		
DISTRIBUTION/AVAILABILITY CODES		
STK.	AVAIL. AND/OR SPECIAL	
A		

UNCLASSIFIED

SECURITY CLASSIFICATION OF THIS PAGE(When Data Entered)

Biological Activities of the Peptides of Staphylococcal
Enterotoxin C Formed by Limited Tryptic Hydrolysis^{1,2}

LEONARD SPERO AND BEVERLY A. MORLOCK

From the U.S. Army Medical Research Institute of Infectious Diseases
Fort Detrick, Frederick, Maryland 21701

Short Title: Biological Activity of Peptides of Enterotoxin C

Approved for public release; distribution unlimited.

Clearance date: 8 May 1978

Biological Activities of the Peptides of Staphylococcal Enterotoxin C
Formed by Limited Tryptic Hydrolysis^{1,2}

LEONARD SPERO AND BEVERLY A. MORLOCK

From the U.S. Army Medical Research Institute of Infectious Diseases

Fort Detrick, Frederick, Maryland 21701

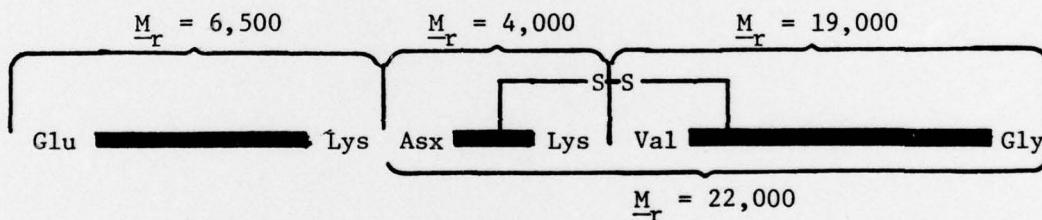
Short Title: Biological Activity of Peptides of Enterotoxin C

SUMMARY

Staphylococcal enterotoxin C₁ is converted to a doubly cleaved molecule by trypsin digestion with one of the scissions internal to the disulfide loop and one external to it [J. Biol. Chem. 251, 5580-5588 (1976)]. The larger, disulfide-containing polypeptide ($M_r = 22,000$) exhibited excellent binding to antiserum to the intact enterotoxin. The residual amino terminal fragment ($M_r = 6,500$) also bound to this antibody but only weakly. Only the carboxyl terminal carboxamidomethylated moiety of the 22,000 M_r polypeptide ($M_r = 19,000$) combined with anti-enterotoxin C₁. Both the 22,000 M_r and 6,500 M_r polypeptides could partially inhibit the binding of enterotoxin C₁ to its antibody in a competitive system. It is suggested that enterotoxin C₁ possesses three major antigenic determinants, two on Cam 19,000 and one on the 6,500 M_r fragment. A significant degree of refolding to a native-like conformation is indicated for the 22,000 M_r and the Cam 19,000 materials by (a) the strong binding of these polypeptides to anti-enterotoxin C₁; (b) the strong binding of enterotoxin C₁ to antibody to the 22,000 M_r polypeptide; and (c) their circular dichroic spectra in the far-ultraviolet.

The 6,500 M_r polypeptide exhibited mitogenic activity but not emetic activity. Conversely the 22,000 M_r polypeptide was able to induce diarrhea in rhesus monkeys, but was not mitogenic suggesting that the active sites for these activities are widely separated on the enterotoxin molecule.

Two of the enterotoxins elaborated by certain strains of Staphylococcus aureus, enterotoxin B and enterotoxin C₁, have been shown to be susceptible to limited specific enzymic digestion by trypsin (1, 2). The primary cleavage in both instances occurs at a site on the polypeptide chain interior to the disulfide loop. A secondary cleavage also occurs which for enterotoxin C₁ goes rapidly to completion. The doubly cleaved molecule, enterotoxin C₁-T₂, may be represented in schematic linear form where the indicated molecular weights were obtained from amino acid analysis:



Both enterotoxin B-T and enterotoxin C₁-T₂ retain all the biological activities of the parent toxins: binding to antibody, mitogenicity, and the induction of emesis and diarrhea. Moreover, enterotoxin C₁-T₂ behaves as a single particle with essentially unaltered conformation.

The extraordinary lability to limited tryptic hydrolysis does not appear to play a role in enterotoxicity. Although the site of nicking is in the disulfide loop and thus bears a structural similarity to that occurring with several of the bacterial exotoxins (3, 4, 5) a parallel biological activation does not take place (1). We have suggested (2) that the kind of lability exhibited by these enterotoxins is associated with β turn structures at the protein surface. The labile Lys-Thr bond in enterotoxin B was found by the Chou and Fasman procedure for the

prediction of secondary structure (6) to be part of such a region. It was noted too that a naturally nicked bond in concanavalin A was placed by X-ray crystallography between the second and third residues of an exposed β turn (7). A similar situation with human β_2 -microglobulin has come to our attention (8). A Tyr-Ser bond at positions 10-11 is unusually susceptible to cleavage by chymotrypsin. Application of the Chou and Fasman method indicates that this serine residue is the first member of a tetrapeptide with a high probability for forming a β turn. These specific cleavages can provide a means of obtaining large well-defined polypeptide fragments and we report here on the association of the polypeptides of enterotoxin C₁ with the various biological activities of the parent enterotoxin. It is demonstrated that both major tryptic polypeptides of enterotoxin C₁-T₂ possess antigenic determinants but that mitogenic activity is restricted to one fragment and emetic activity to the other.

EXPERIMENTAL PROCEDURES

Materials -- Staphylococcal enterotoxin C_1 and C_1-T_2 were prepared as previously described (2). The 22,000 molecular weight and 6,500 molecular weight polypeptides of enterotoxin C_1-T_2 were separated on a column of Sepharose 6B (Pharmacia) in 6 M guanidine hydrochloride (Schwarz/Mann, Ultrapure grade) (2). For some testing of biological activity it was necessary to purify these two polypeptides further. The 6,500 M_r polypeptide was rechromatographed under the same conditions. The single resultant peak was dialyzed free of guanidine and passed through an affinity column of rabbit antibody to enterotoxin C_1 bound to Sepharose 4B (9). This was to remove any residual trace of intact enterotoxin C_1-T_2 reformed by complementation of contaminating 22,000 M_r polypeptide with the 6,500 M_r fragment. The 22,000 M_r polypeptide, although appearing as a single peak in the gel filtration used for its isolation, was significantly contaminated with enterotoxin C_1-T_1 . Rechromatographing this fraction three times, each time selecting the latter 1/2 to 2/3 of the peak served to reduce the contamination to levels acceptable for assay.

After reduction with β -mercaptoethanol and alkylation with iodoacetamide, the 22,000 molecular weight polypeptide was separated into its constituent peptides, Cam³ 4,000 and Cam 19,000 by chromatography on Sepharose 6B in 6 M guanidine hydrochloride. These reactions were carried out as previously described (2) except that the medium was 6 M with respect to guanidine hydrochloride.

Preparation of Antisera -- Anti-enterotoxin C_1 was prepared by intracutaneous injection of the enterotoxin without adjuvant in New Zealand white rabbits⁴. A regimen based on that developed by Silverman

(10) was employed. Rabbit antiserum for the 22,000 M_r polypeptide was produced by intramuscular inoculation of three 100 μ g doses of polypeptide at weekly intervals in 10% rabbit serum albumin. Most of the serum used in this study was from a bleeding made one week after a second course of immunization administered 3 months after the first series of injections.

Labeling of Enterotoxin C₁ and Tryptic Peptides -- All labeling was carried out by the gaseous diffusion method of Gruber and Wright (11) with ¹²⁵I. Enterotoxin C₁ was labeled in phosphate buffered saline solution. The peptides were dissolved in 6 M guanidine hydrochloride during labeling and unbound radioisotope was removed by dialysis against 6 M guanidine hydrochloride.

Antigen-binding Capacity and Competitive Binding Assay -- These techniques are based on the ability of protein A-containing strains of S. aureus to react specifically and with high affinity with the Fc portion of IgG (12). In the antigen-binding capacity assay samples of labeled antigen (25 to 100 ng in 500 μ l) were added to 500 μ l volumes of twofold, serial dilutions of antiserum. Dilutions of enterotoxin were made in phosphate buffered saline containing 0.5% bovine serum albumin. For binding assays with the peptides the antiserum dilutions were prepared in buffered saline containing 10% albumin. To separate bound from unbound antigen a 10% suspension of the Cowan I strain of S. aureus was added. For serum dilutions of 1:40 and above, 100 μ l of the 10% suspension were adequate to precipitate all the immunoglobulin but for dilutions of 1:10 and 1:20, 500 μ l were required and for 1:5 dilutions of serum a full milliliter was needed. The reaction tubes were treated and the calculation of percentage of antigen bound to

antibody was made as previously described (13). In the calculations a correction was made for nonspecific precipitation by the bacterial cell suspension (13).

In the competitive binding assay, a volume of antiserum sufficient to bind 50% of 100 ng of labeled enterotoxin C₁ was added to varying amounts of unlabeled inhibitor and the solutions were incubated at room temperature for 20 min. The homologous labeled antigen was added and after an overnight incubation at 4° the tubes were processed with the 10% suspension of S. aureus as described above.

Other Methods -- The extent of the contamination of the 6,500 M_r polypeptide and the 22,000 M_r polypeptide with materials of the molecular size of enterotoxin C₁ was estimated by gel electrophoresis in Na dodecyl-SO₄. With this procedure contamination of less than 1 part in 1,000 is detectable, since samples of about 100 µg were applied to the gels and the presence of 0.1 µg is easily observed. Standards up to 0.5 µg gave a linear response with the area of the stained zone, after scanning with a Gilford Model 2410-S linear transport device.

Methods for the estimation of emetic activity and of mitogenic activity have been described (1, 14). Circular dichroic spectra were obtained on a Jasco J-40 instrument with a data processor. Peptides were prepared for analysis by dialysis of concentrated solutions in 6 M guanidine hydrochloride against repeated changes of large volumes of phosphate buffered saline. Concentrations of 1 mg/ml were readily achieved for the 6,500 M_r polypeptide and Cam 4,000. However, extensive precipitation occurred with both Cam 19,000 and the 22,000 M_r polypeptide. These solutions were clarified by centrifugation;

a concentration of 0.6 mg/ml was obtained with Cam 19,000 but only 0.4 mg/ml with the 22,000 M_r fragment. At least 32 repetitive scans were run using the data processor at a time constant of 4 s. A cell with a path length of 0.1 mm was used in the far-ultraviolet for all the peptides except the 22,000 M_r material where a 0.5-mm path length was employed.

RESULTS

Solubilization of Peptides -- Obtaining the peptides in a stable, soluble state from the concentrated guanidine hydrochloride solutions in which they were isolated was a significant problem especially for the 22,000 M_r fragment. Dialysis against aqueous buffers over a wide pH range resulted in precipitation of most of the polypeptide and an unstable solution. It was soluble in both anionic (Na dodecyl-SO₄) and cationic (cetylpyridinium chloride) detergent solutions but serologic activity could not be demonstrated in the presence of either detergent. Successful solubilization was achieved by dilution into concentrated solutions of bovine serum albumin. The general procedure consisted of the dropwise addition of a 5 to 10 mg/ml solution of the polypeptide in 6 M guanidine hydrochloride [obtained by dry concentration with Aquacide (Calbiochem)] to a vigorously stirred 10% buffered solution of the albumin. Concentrations up to 1 mg/ml were readily obtained and the solutions were stable for several days. Except where noted all biological measurements were carried out on peptides prepared in this manner.

Serologic Properties of the Tryptic Peptides -- The binding capacities of the radiolabeled major peptides of enterotoxin C₁-T₂, the 6,500 M_r fragment and the 22,000 M_r fragment, are compared in Fig. 1 with that for intact [¹²⁵I]enterotoxin C₁. The data have been normalized to equal amounts of labeled antigen; this is based on the assumption that over the range of antigen employed the ratio of antibody to antigen at the endpoint is independent of antigen level. It is readily apparent that the 22,000 M_r polypeptide bound very well to the antibody to the whole enterotoxin; on a weight basis the affinity

was nearly one-half that of enterotoxin C₁, and essentially all of the label was precipitable. The binding of the 6,500 M_r polypeptide was significant but considerably weaker.

Competitive inhibition of these two polypeptide fragments with the native enterotoxin is presented in Fig. 2. Both materials competed successfully with the native toxin for its homologous antibody. As anticipated, the 22,000 M_r polypeptide was the more potent inhibitor but a plateau of reaction was not reached with either fragment at its highest available concentration. If it is assumed that each fragment contained a unique determinant, some antibodies for intact toxin would be incapable of combining with the fragment under assay and total inhibition of the native enterotoxin-antiserum reaction could not be achieved.

Neither of these polypeptide preparations contained 28,000 M_r material by polyacrylamide gel electrophoresis. However, as noted earlier, the detectable limit of the method is slightly better than 0.1%. This does not present a problem for the 22,000 M_r polypeptide because significant inhibition was observed at concentrations where contamination levels with whole enterotoxin of 1% would be necessary to produce the observed effect. Moreover, if the inhibitory response were indeed due to contamination, the inhibition curve would have been identical to that seen with enterotoxin C₁ but displaced to the right (cf ref. 13, Fig. 6). With the 6,500 M_r polypeptide we must rely on the methods employed in its isolation and purification; after the second chromatographic separation 28,000 molecular weight material was not detected on analytic gels and subsequent purification with the antibody affinity column was demonstrated to be capable of removing enterotoxin contaminants several orders of magnitude greater than would be present.

These difficulties are obviated in the antigen-binding capacity assay where trace contamination cannot contribute significantly to the percent of labeled antigen bound to the antibody. Binding data with antisera to enterotoxin C₁ and to the 22,000 M_r polypeptide are shown in Table I for intact enterotoxin and its four tryptic peptides. In these calculations it was assumed that all the antigen bound at the 50% endpoint was in the form Ag₂Ab (15) so that for the bivalent antibody the molar ratio of antigen to antibody was 4. Endpoints were estimated from log-log plots of the volume of antibody against the percent of antigen bound. These graphs were linear from about 20 to 80% bound and had nearly identical slopes. This latter property facilitated the endpoint estimation when an extrapolation from the data was required.

These molar ratios emphasize the contrast between the excellent binding of the 22,000 M_r polypeptide and the comparatively weak binding of the 6,500 M_r polypeptide to anti-enterotoxin C₁. Lack of binding of Cam 4,000 to this antiserum indicated the absence of a native determinant on this portion of the 22,000 M_r fragment. In contrast, Cam 19,000 bound very well to the antiserum requiring only six times as much antibody as the whole enterotoxin. It was, however, less efficacious than the 22,000 M_r polypeptide. This may be attributed to either the absence of a determinant or to inferior restoration of native structure in the somewhat smaller polypeptide. The lack of binding by Cam 4,000 leads us to favor the latter explanation.

The excellent binding of the 22,000 M_r polypeptide to anti-enterotoxin C₁ is indicative of a high degree of refolding of this fragment to a native conformation. This contention is supported by the

surprisingly strong binding of the whole enterotoxin to antiserum raised against the 22,000 M_r polypeptide. Enterotoxin C₁ is a very stable, compact protein and the antibody sites which it combines reflect immunoglobulin biosynthesis induced by determinants intrinsic to the intact native enterotoxin, i.e., these regions must also be present in the immunogen, the 22,000 M_r polypeptide. It is well established that antibodies elicited by immunization with denatured protein either fail to react, or do not react extensively, with the native protein (16). Conversely it is also clear that complete refolding does not exist in the 22,000 M_r polypeptide. This was demonstrated by (1) the binding of Cam 4,000 for the antibody to the 22,000 M_r polypeptide despite its complete failure to react with anti-enterotoxin C₁ and (2) by the better binding of Cam 19,000 to the anti-22,000 M_r polypeptide than to anti-enterotoxin C₁.

Emetic Activity of the Tryptic Peptides of Enterotoxin C₁ -- All the animals used for test of emetic activity of the tryptic polypeptides had no antibody titer to enterotoxin B or C₁ by hemagglutination assay. No animals died and all appeared to be completely normal within 24 hours after inoculation. The 6,500 M_r polypeptide produced no emesis or diarrhea in monkeys injected intravenously with doses up to 10 $\mu\text{g}/\text{kg}$, the equivalent to 300 median effective (ED_{50}) doses of the intact enterotoxin. The results of assay of the 22,000 M_r polypeptide in rhesus monkeys are shown in Table II. This preparation of the polypeptide contained 0.25% contamination with 28,000 M_r material which represents less than one-fourth of an ED_{50} of enterotoxin at the higher dose of the 22,000 M_r polypeptide at which diarrhea occurred. A positive response to this level of enterotoxin C₁ has not been observed.

A positive result at the same level was obtained with another preparation of the 22,000 M_r polypeptide in cynomolgus monkeys. It must be noted that no emesis was seen with this large polypeptide and the diarrhea was always less severe than with intact enterotoxin. We concluded that the active site for emesis and diarrhea is located within that portion of the molecule isolated on the 22,000 M_r polypeptide. The greatly reduced activity may be due to any of several factors operating singly or in combination, e.g., faster metabolic turnover, impaired conformation, or weaker binding to the putative receptor.

Mitogenic Activity of the Tryptic Peptides of Enterotoxin C₁ --

The 6,500 M_r polypeptide demonstrated a low level of mitogenic activity. A typical response is shown in Fig. 3. In these experiments the 6,500 M_r polypeptide was prepared by removal of the guanidine in which it was isolated by dialysis against phosphate buffered saline. No mitogenic activity was found for the 22,000 M_r polypeptide under any of several solubilized conditions.

Circular Dichroism of the Peptides -- In Fig. 4 are presented the circular dichroic spectra of the four tryptic peptides in the far-ultraviolet. The spectrum of the native enterotoxin (2) in that region is included for comparison. The curves for the 6,500 M_r polypeptide and for Cam 4,000 are typical of random coil conformation. Both Cam 19,000 and the 22,000 M_r fragments, however, show significant similarity to that of the intact enterotoxin. The spectrum of enterotoxin C₁ is very much like that of enterotoxin B which has been reported by analysis of its circular dichroism and by the Chou and Fasman procedure for the prediction of secondary structure (6) to

contain about 30% β -pleated sheet and about 10% α -helix (17). A striking feature of this structure is extensive grouping of anti-parallel β -pleated sheet around the disulfide loop. Much of this would be retained in both of these large polypeptides.

DISCUSSION

Quantitative precipitin analysis of enterotoxin C₁ gives an effective antigen valence of 3 to 4. We assume for the purposes of discussion that the native molecule has three major antigenic determinants. Assay of antigen binding capacity with anti-enterotoxin C₁ demonstrated that the presence of determinants (a) on the amino terminal 6,500 M_r polypeptide, (b) on the 22,000 M_r polypeptide, and (c) on Cam 19,000 (the carboxyl terminal moiety of the 22,000 M_r polypeptide), but not on Cam 4,000 (the amino acid terminal moiety of the 22,000 M_r polypeptide). In competitive inhibition assays a greater than 60% inhibition was achieved by the 22,000 M_r polypeptide and 21% inhibition by the 6,500 M_r polypeptide suggesting that the larger polypeptide can combine with two-thirds of the antibody population and the smaller polypeptide with one-third of the antibody population. It is further inferred that the 22,000 M_r polypeptide possesses two determinants (restricted to Cam 19,000) and the 6,500 M_r polypeptide one determinant.

This interpretation is consistent with our failure to find precipitation by the 22,000 M_r polypeptide with anti-enterotoxin C₁ in either free solution or in gel diffusion (data not shown). It does not preclude the presence of an additional determinant on this polypeptide, only that such a determinant is not formed when the fragment is removed from the denaturing environment of 6 M guanidine hydrochloride by dilution into serum albumin. A similar caveat must be considered for the lack of binding of Cam 4,000 to antiserum to the native enterotoxin. This fragment may conceivably require the presence of additional segments of the polypeptide chain in order to form a

conformational determinant. The concept of a "native format determinant" put forth by Sachs et al. (18) in which all the components of a determinant are present in a limited length of the polypeptide chain "but exist in solution in equilibrium in between a variety of disordered conformations," may be comfortably applied to the present results. It can account for the very low binding of the 6,500 M_r polypeptide relative to the binding of the 22,000 M_r polypeptide. In addition it can explain the binding of the 6,500 M_r polypeptide to anti-enterotoxin C₁ despite the lack of secondary structure apparent in its circular dichroic spectrum. It is also applicable to the superior binding of the 22,000 M_r polypeptide compared to Cam 19,000 to both antisera employed in this study. The overall binding constant is considered to be the product of two factors, an association constant, and a conformational equilibrium constant. If both polypeptides possess the same binding sites the association constants would be equivalent, and the difference in overall binding would be due to a difference in conformational constants. The very excellent binding of the 22,000 M_r polypeptide and the good binding of Cam 19,000 to the antibodies to enterotoxin C₁ suggest that the equilibria for these fragments are greatly in favor of the native structure. This is supported biologically by the observation that native enterotoxin binds extremely well to antibody to the 22,000 M_r fragment. It is supported also by the physical properties reflected in the circular dichroic spectra in the far-ultraviolet, which indicate that both polypeptides have a similar secondary structure to the whole enterotoxin. It may be noted in passing that this also indicates that the primary nucleation site for folding is in this part of the amino acid sequence. Further, it

is contrary to the idea that virtually an entire sequence is required for folding to a native conformation from a disordered state (19).

Wetlaufer and co-workers have obtained a native-like structure from the peptide 13-105 of hen egg lysozyme (20).

The assays for mitogenic and emetic activity of the two major polypeptides of enterotoxin C₁-T₂ indicate that these activities are associated with widely separated regions of the polypeptide chain.

Mitogenesis was induced by the amino terminal fragment, and diarrhea by the remaining portion of the molecule. We earlier suggested (14) that a residual mitogenic activity of enterotoxin B, detoxified by treatment with formaldehyde, implied that the mitogenic and emetic sites were not identical. Our present studies on enterotoxin C₁ tend to confirm this and to provide a general localization of the sites responsible for these two activities.

REFERENCES

1. Spero, L., Warren, J. R., and Metzger, J. F. (1973) J. Biol. Chem. 248, 7289-7294.
2. Spero, L., Griffin, B. Y., Middlebrook, J. L., and Metzger, J. F. (1976) J. Biol. Chem. 251, 5580-5588.
3. Collier, R. J. (1975) Bacteriol. Rev. 39, 54-85.
4. DasGupta, B. R. (1971) J. Bacteriol. 108, 1051-1057.
5. Matsuda, M., and Yoneda, M. (1974) Biochem. Biophys. Res. Commun. 57, 1257-1262.
6. Chou, P. Y., and Fasman, G. D. (1974) Biochemistry 13, 222-245.
7. Becker, J. W., Reeke, G. N., Jr., Wang, J. L., Cunningham, B. A., and Edelman, G. M. (1975) J. Biol. Chem. 250, 1513-1524.
8. Cunningham, B. A. (1976) Fed. Proc. 35, 1171-1176.
9. Cuatrecasas, P., Wilchek, M., and Anfinsen, C. B. (1968) Proc. Natl. Acad. Sci. U.S.A. 61, 636-643.
10. Silverman, S. J. (1963) J. Bacteriol. 85, 955-956.
11. Gruber, J., and Wright, G. G. (1969) J. Bacteriol. 99, 18-24.
12. Forsgren, A., and Sjöquist, J. (1966) J. Immunol. 97, 822-827.
13. Spero, L., Morlock, B. A., and Metzger, J. F. (1978) J. Immunol. 120, 86-89.
14. Spero, L., Leatherman, D. L., and Adler, W. H. (1975) Infect. Immun. 12, 1018-1020.
15. Osler, A. G. (1971) in Methods in Immunology and Immunochemistry (Williams, C. A., and Chase, M. W., eds) Vol. III, pp 73-85, Academic Press, New York.

16. Crumpton, M. J. (1974) in The Antigens (Sela, M., ed.) Vol. II, pp 1-78, Academic Press, New York.
17. Muñoz, P. A., Warren, J. R., and Noelken, M. E. (1976) Biochemistry 15, 4666-4671
18. Sachs, D. H., Schechter, A. N., Eastlake, A., and Anfinsen, C. B. (1972) Proc. Natl. Acad. Sci. U.S.A. 69, 3790-3794.
19. Anfinsen, C. B. (1961) J. Polymer Sci. 49, 31-49.
20. Johnson, E. R., Anderson, W. L., Wetlaufer, D. B., Lee, C.-L., and Atassi, M. Z. (1978) J. Biol. Chem. 253, in press.

Footnotes

- ¹ Presented in part at the 67th Annual Meeting of the American Society of Biological Chemists, San Francisco, Calif., June 1976.
- ² The views of the authors do not purport to reflect the position of the Department of the Army or the Department of Defense.
- ³ Abbreviation used: Cam, carboxamidomethyl.
- ⁴ In conducting the research described in this report, the investigators adhered to the "Guide for the Care and Use of Laboratory Animals," as promulgated by the Committee on the Revision of the Guide for Laboratory Animal Facilities and Care of the Institute of Laboratory Animal Resources, National Research Council. The facilities are fully accredited by the American Association for Accreditation of Laboratory Animal Care.

Acknowledgments -- We wish to thank Mr. D. L. Leatherman for the mitogenicity assays and Dr. J. G. Miller for help with the assays for emetic activity.

Table I

Binding of enterotoxin C₁ and tryptic peptides derived from it
with antisera

The antigens were labeled and binding capacity determined as described in Experimental Procedures. Fifty percent endpoints were estimated from log-log plots of the volume of antibody added against the percent of antigen bound. Antibody levels and molar ratios were calculated assuming that all antigen bound at the endpoint was in the form of Ag₂Ab.

Antigen	Molar ratio of total antibody to labeled antigen at 50% endpoint	
	Anti-22,000 M _r	Anti-enterotoxin C ₁
Enterotoxin C ₁	0.57	0.25 ^a
6,500 M _r	NBE ^b	81 ^c
22,000 M _r	0.25 ^a	0.44
Cam 4,000	4.3 ^c	NBE
Cam 19,000	1.2	1.5

^a Fixed by computational assumptions.

^b No binding evident.

^c Endpoint estimated by linear extrapolation of log-log plot.

Table II

Emetic activity of 'enterotoxin C₁ and 22,000 M_r polypeptide

Rhesus monkeys (Macaca mulatta; weight approximately 3 kg) were injected intravenously in the saphenous vein. The animals were observed continuously for 5 hr after administration of the toxin.

Dose μg/kg	Enterotoxin C ₁			22,000 M _r Polypeptide		
	Diarrhea and emesis	Latent period	Cumulative ^a experience	Diarrhea	Latent period	Total
	No. positive/Total	min	No. positive/ Total	No. positive/Total	min	
0.003	-		0/4	-	-	
0.01	-		0/8	-	-	
0.03	3/4	148, 198, 235	13/17	-	-	
0.1	3/4	82, 114, 143	15/17	0/4	-	
0.3	1/3	112	12/17	2/4	63, 123	
1.0	-		8/8	-	-	
3.0	-		2/4	57, 154		

^a Includes present data

Legends for Figures

Fig. 1. Binding of ^{125}I -labeled enterotoxin C_1 and of two ^{125}I -labeled polypeptides derived from it by trypsin digestion to rabbit antiserum to enterotoxin C_1 . The data have been normalized to equal amounts of labeled antigen. ●, enterotoxin C_1 ; □, 22,000 M_r polypeptide; ○, 6,500 M_r polypeptide.

Fig. 2. Inhibition of binding of $[^{125}\text{I}]$ enterotoxin C_1 to rabbit antiserum to enterotoxin C_1 . Inhibitors used were: ●, enterotoxin C_1 ; □, 22,000 M_r polypeptide; ○, 6,500 M_r polypeptide.

Fig. 3. Mitogenic effect of enterotoxin C_1 and 6,500 M_r polypeptide derived from it by trypsin digestion. C57BL/6 mouse spleen cells ($2.0 \times 10^6/0.5 \text{ ml}$) were incubated in Roswell Park Memorial Institute medium 1640 supplemented with 5% human serum and the indicated amount of mitogen for 48 hr under 5% CO_2 -95% air at 37° . A pulse of 0.5 μCi of $[^3\text{H}]$ thymidine was added for 4 hr. ●, enterotoxin C_1 ; ○, 6,500 M_r polypeptide.

Fig. 4. Circular dichroic spectra in the far-ultraviolet of enterotoxin C_1 and the polypeptides derived from it by trypsin digestion.
—, enterotoxin C_1 ; ·—·, 22,000 M_r polypeptide; ——, Cam 19,000; -----, 6,500 M_r polypeptide; ..—.., Cam 4,000.

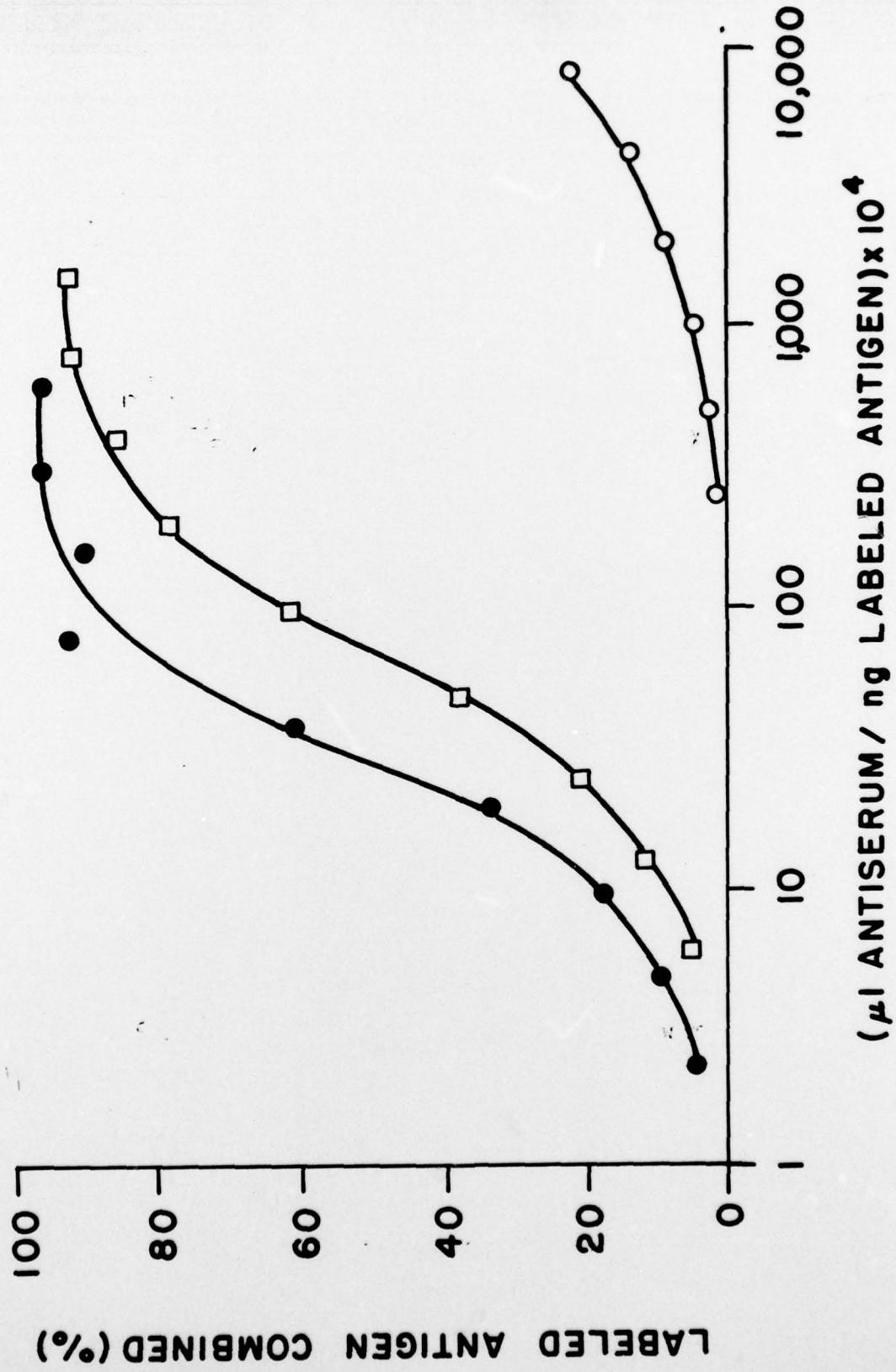
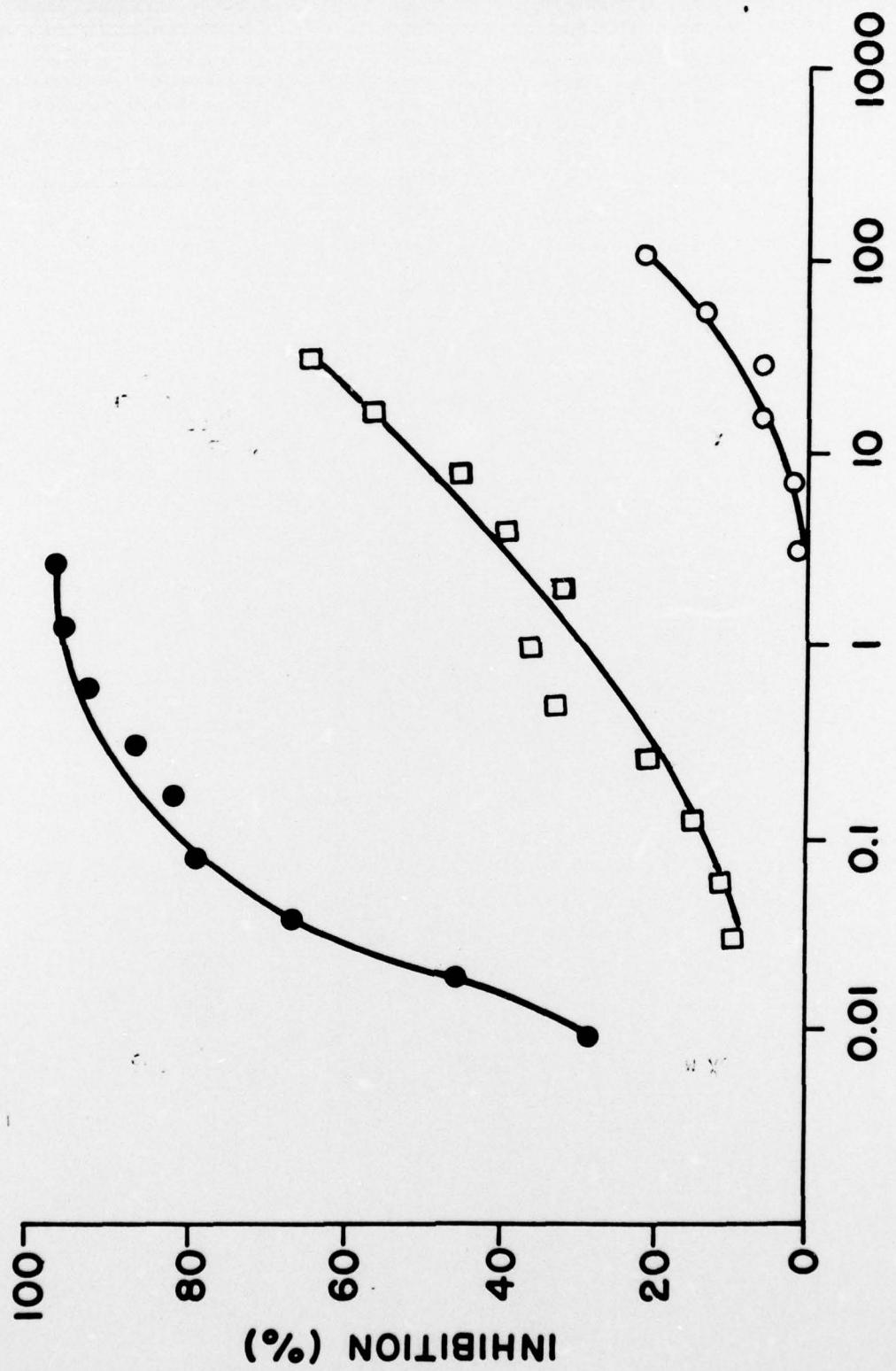


Figure 1

INHIBITOR ADDED (μ g)

Figure 2



MITOGEN ADDED (μ g)
Figure 3



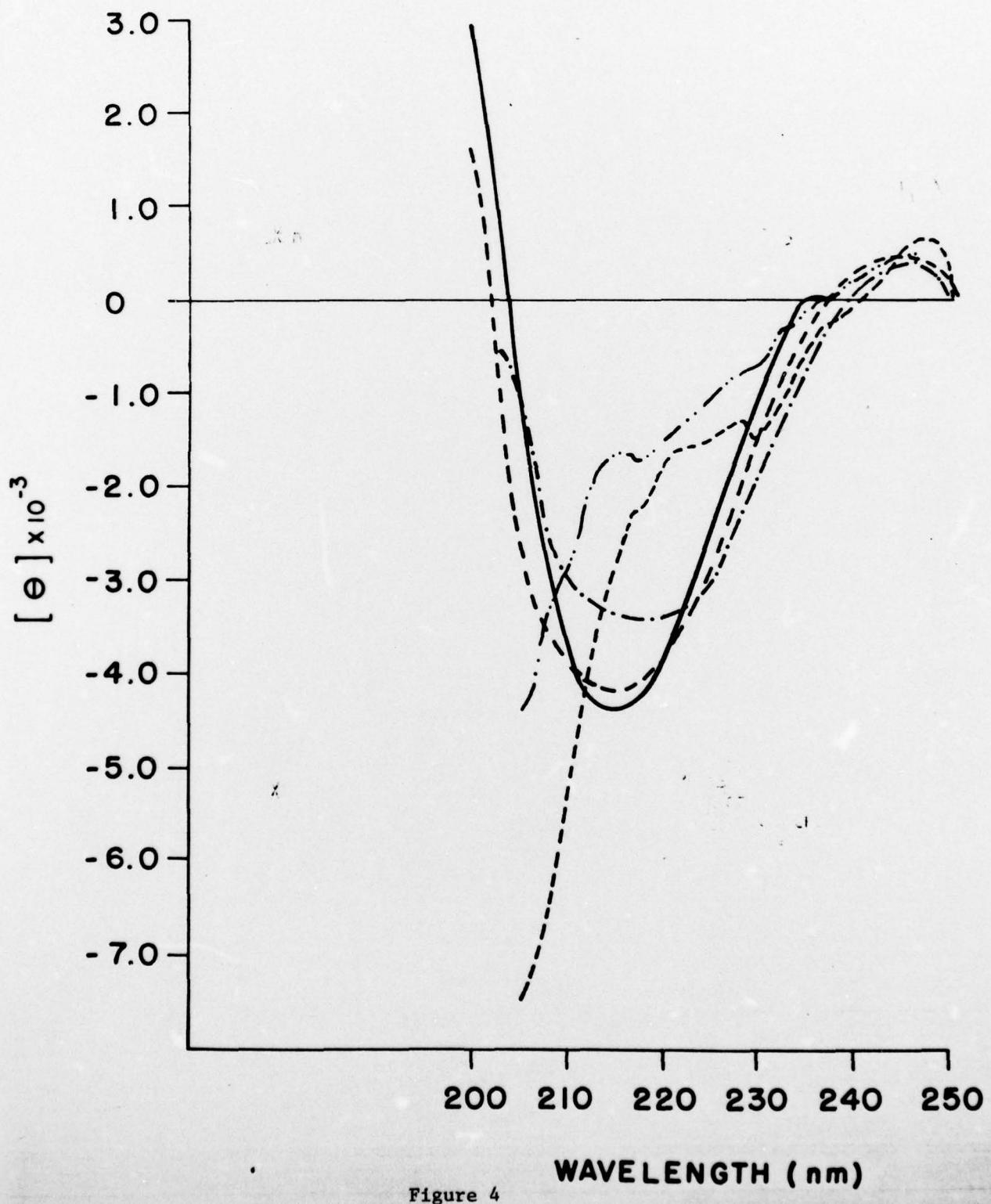


Figure 4